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13. ABSTRACT (Maximum 200 Words)

Our laboratory has recently synthesized a series of novel substituted indanones that are selectively toxic to multidrug resistant cancer cells, including breast cancer cell lines.

In this application we proposed to characterize the mechanism of action of indanocine and to assess the in vivo anti-tumor activity of indanocine.

During the second year we:
- published the second report on the biological activity of indanocine (Cancer Res 2001 Oct 1;61(19):7248-54)
- analyzed the indanocine-resistant stable cell line
- identified the potential indanocine-binding site on tubulin
- continued the animal testing of indanocine
- studies the pro-apoptotic mechanism of action in non-dividing tumor cells

The results shown in this annual summary demonstrate that indanocine is a very promising new anti-cancer agent, with selective activity in slowly-dividing or quiescent tumor cells. The positive early animal models suggest that indanocine could be soon ready for clinical trials

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Annual Award Report, Government Award Number DAMD17-99-1- 9100: Mechanism of Action of Substituted Indanones in Multidrug Resistant Breast Cancer

Our laboratory has synthesized a series of novel substituted indanones that are selectively toxic to multidrug resistant cancer cells, including breast cancer cell lines.
In this application we proposed to characterize the mechanism of action of indanocine and to assess the in vivo anti-tumor activity of indanocine.

During the first two years we:
- published the first report on the biological activity of indanocine (J Natl Cancer Inst 2000, 92:217-224)
- generated an indanocine-resistant stable cell line and identified the binding site on beta-tubulin (Cancer Res 2001, 61:7248-54)
- demonstrated the selective activity of indanocine-phosphate against primary tumor cells and proposed a novel mechanism of action for stationary tumor cells (Manuscript submitted to Blood and in review)
- synthesized a water-soluble analog of indanocine (indanocine-phosphate) and several analogs
- tested the anti-angiogenic activity of indanocine-phosphate in *in vivo* animal models
- demonstrated the anti-tumor activity of indanocine-phosphate in *in vivo* animal models
- obtained results in a serie of animal models in collaboration with the National Cancer Institute
- obtained an issued patent on the composition of matter of indancine and related analogs
- licensed the patent to a start-up company that is currently developing indanocine and related analogs for clinical trials that should start in 2004

Given all of the listed accomplishments, the decision of the reviewer to reject of second year report came as a surprise. We do understand that some of the some relevant results obtained with the support of this DOD grant were on cells other than breast cancer, but the change in the experimental plan were dictated by the initial results obtained, We believe that we may have failed in our effort of explaining the reasons of these changes in previous reports.

We hope to be able to demonstrate in this new updated report why we believe that the reviewer's rejection is unfair and to illustrate the reasons why we decided to perform the experiments listed in the progress report.

For the benefit of the reviewers I included at the end of this update the previous reports that were submitted to the attention of the DOD. Taken together, including the published papers and the patent, this document includes all the results obtained up to the end of 2002 with the support of the DAMD17-99-1-9100 grant.

Comments to Task 1

The reviewer indicated that none of the tasks outlined in the proposal have been initiated. We believe that this statement is unmerited and to prove this we would like to recapitulate the tasks:

Task 1
In the first aim of our application, we proposed to characterize further the mechanism of action and the molecular targets of indanocine and related compounds in multidrug resistant breast cancer cell lines. Specifically, we planned to:

1. Develop indanocine resistant breast cancer cell line mutant and assess its pattern of cross resistance to other agents (months 1-24)
2. Characterize biochemically the resistant phenotype, emphasizing cytoskeletal and apoptotic regulatory proteins (months 7-18)
3. Investigate differentially expressed genes in the resistant mutants by human cDNA expression chips (months 12-24)
4. Test the role of the differentially expressed genes by transfection in the wild type cell lines (months 24-32)

Comments: We have generated a cell line resistant to indanocine, described the mechanism of resistance, identified the mutation in the beta-tubulin gene responsible for the resistance and mapped a novel tubulin-binding site for anti-mitototic agents (Cancer Res 2001, 61:7248-54). Unfortunately, for the reviewer, the line we used was from leukemic origin. We failed in our repeated attempts to generate breast cancer lines resistant to indanocine. We employed the same procedures described in the Cancer Research paper (and described originally in our grant proposal) with MCF7, MDA-MB231, T47D and others with no avail. We do not understand the reasons for our failure in developing indanocine-resistant breast cancer cells, but we have reason to believe that the mechanism of resistance described in our manuscript may eventually occur in breast cancer cells. In fact, beta-tubulin is abundantly expressed in breast cancer cell lines and mutations could occur in any cancer types. As for task, 1.3 and 1.4, the identification of the principal mechanism of resistance (mutation of the beta-tubulin) rendered the gene-expression analysis portion of the task redundant.

One of the unexpected major findings we obtained studying the effect of indanocine in breast cancer cells (J Natl Cancer Inst 2000, 92:217-224) was the pro-apoptotic activity of the compound in stationary, non-dividing multidrug resistant cells. This major finding was emphasized in an editorial published in J. National Cancer Institute (J Natl Cancer Inst 2000 92:182-3). Therefore, we decided to initiate some additional experiments to understand the mechanism of action of indanocine in this type of cells. An ideal source of primary, stationary, drug-resistant tumors is chronic lymphocytic leukemia (CLL) cells isolated from CLL patients. We investigated the mechanism of action of indanocine in these cells, and elucidated a pro-apoptotic signaling pathway that appears to be novel and may be relevant for the killing of non-dividing breast cancer cells. The manuscript submitted for publication to Blood is available upon request. The initial reviews of this paper were excellent and we have reasons to believe that the manuscript will be soon accepted. Further experiments will be carried out to explore if the observed mechanism can be recapitulated in breast cancer cells. We fully understand that these experiments represent a diversion from the proposed research plan, but we hope the reasons for this change of plans will be now more understandable.

Comments to Task 2

In the second aim of the application we proposed to assess the in vivo antitumor activity of indanocine in nude mice bearing multidrug resistant human breast cancer cell lines. Specifically, we planned to:
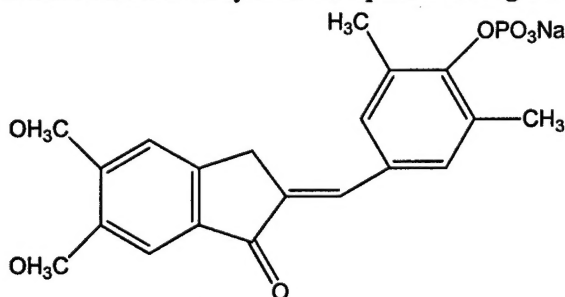
1. Determine the acute and chronic toxicity of indanocine and related compounds in nude mice.
2. Establish human tumor models in nude mice using wild type, multidrug resistant, and indanocine resistant breast cancer cell lines.
3. Test indanocine and related compounds in the established in vivo tumor models

Comments: The very first assays with Indanocine revealed that the compound was very poorly active in several animal model tested. One of the major problem was that Indanocine is very poorly water soluble and needed to be formulated for delivery in animals (as already reported in previous reports). To address these issues we decided to use two approaches: generation of a water soluble phosphor-ester pro-drug (Indanocine phosphate), generation of chemical analogs of Indanocine with improved in vivo activity profile. These approaches required time and considerable effort, but testify our eagerness in continuing the development of the project and accomplish the proposed tasks. The reviewers should take this issues in consideration when evaluating our results.

A) Results with Indanocine phosphate

Preliminary results obtained with Indanocine-phosphate (see structure below, abbreviated as SDX-103) were described in the previous reports, suggesting an anti-angiogenic and anti-vascular activity.

A more detailed analysis of the optimal dosing schedule and the maximal tolerated dose for

Indanocine phosphate is included in this report (table 1 and 2). The results of the initial anti-tumor efficacy study using a wild-type and a multidrug resistant tumor model is also included (Table 3 and 4, Figure 1 and 2).

In the dose-ranging toxicity studies and MTD was not achieved at doses as high as 300 mg/kg. Several regimens were tested, including a comparison of IP and IV delivery and QD (every day) vs QOD (every 2 days) frequency. We demonstrated that indanocine phosphate is well tolerated by the animals (mice and rats) at doses as high as 300 mg/kg (Table 1) . No mortality or even major weight loss was detectable in animals treated with the compound.

Table 1. Preliminary Toxicity in Atymic Nude Mice. Indanocine-phosphate (SDX-103) was administered

Group Number	Ê N	Regimen 1				Max. BW Loss %; Day	Number of Death	
		Name	mg/kg	Route	Schedule		TR	NTR
1	5	SDX-103	300	IV	QD x 5	-3.0%; Day 4	0	0
2	5	SDX-103	200	IV	QD x 5	---	0	0
3	5	SDX-103	100	IV	QD x 5	-1.4%; Day 11	0	0
4	5	SDX-103	300	IV	QOD x 5	---	0	0
5	5	SDX-103	200	IV	QOD x 5	---	0	0
6	5	SDX-103	100	IV	QOD x 5	---	0	0
7	5	SDX-103	300	IP	QD x 5	---	0	0
8	5	SDX-103	200	IP	QD x 5	---	0	0
9	5	SDX-103	100	IP	QD x 5	---	0	0
10	5	SDX-103	300	IP	QOD x 5	---	0	0
11	5	SDX-103	200	IP	QOD x 5	---	0	0
12	5	SDX-103	100	IP	QOD x 5	-0.5%; Day 8	0	0

to non-tumor bearing female black B6D2F1 atymic nude mice by i.p. and i.v. injections on two schedules (qd x5 and qod x5) in 100, 200, 300 mg/kg doses. No toxicity was observed on any of the regimens. The following in vivo survival studies were then designed to use i.p. administration of SDX-103 at 300 mg/kg and 500 mg/kg.

When then tried to establish breast cancer tumor xenograft at UCSD. Unfortunately, our repeated attempts to establish breast cancer tumor xenografts in athymic mice at were not successful.

For this reason, after these initital dose-ranging experiments, we decided to test the anti-tumor efficacy of indanocine-phosphate in a very well established multidrug resistant syngeneic animal model: the murine lymphoma P388 and the multidrug-resistant subline P388/ADR. We selected these tumor models because they represented a good model for the initial evaluation of this Indanocine prodrug.

The sensitivity of the P388 and P388/ADR cells to Indanocine was verified in vitro by MTT assay. The IC50 were 40±7 nM for P388 and 48±8 nM for P388/ADR, confirming the lack of cross-resistance of Indanocine. For comparison, the IC50 of paclitaxel is >100 fold higher in P388/ADR cells when compared to P388.

The study design for both models is recapitulated in Table 2.

Table 2. Indanocine-phosphate P388 study design. P388 and P388ADR murine tumor cells, cultured in

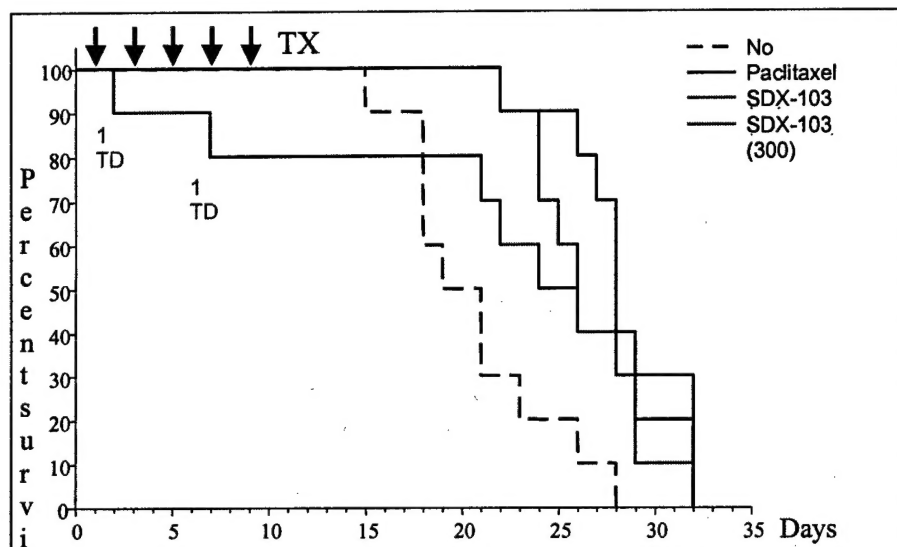| Group	n	Primary				Secondary			
		Agent	mg/kg	Route	Schedule	Agent	mg/kg	Route	Schedule
1	10	No Treatment	n/a						
2	10	Paclitaxel	20	IP	QOD x 5				
3	10	SDX-103	500	IP	D 1, 3, 5	SDX-103	400	IP	D 7, 9
4	10	SDX-103	300	IP	QOD x 5				

RPMI1640 supplemented with 10% FBS, were harvested and resuspended at a concentration of 5×10^6 cells/ml. Each female black B6D2F1 atymic nude mice was inoculated i.p. with 1×10^6 leukemic cells at day 0. Mice were randomized into four groups and treatments were initiated at day 1 as described in the table. Because of acute toxicity in the P388 study group, the group 3 dose (500 mg/kg) was reduced at 400 mg/kg for the last 2 injections at day 7 and 9. The same schedule and doses were used for the P388ADR study, although there were no toxic deaths at the time the three 500mg/kg doses had been delivered. All injections were made in volumes of 0.2 ml/20 g body weight.

The results with the wild type P388 model are reported in Table 3 and Figure 1. We observed a statistically significant anti-cancer effct of indanocine-phosphate when dosed at 300 and 400 mg/kg (at the MTD). The T/C survival ratio was similar to the one observed with paclitaxel, but at a dose that was 20 fold higher.

Group	n	Primary		Secondary		Mean Day of Survival	%T/C	# 30 Day Survival	Max BW. Loss	# of Toxic
		Agent	mg/kg	Agent	mg/kg	Mean ± SEM (n)			%; (Day)	Deaths
1	10	No Treatment	n/a			20.7 ± 1.3 (10)	100%	0	---	0
2	10	Paclitaxel	20			28.1 ± 0.9 (10)	136%*	0	---	0
3	10	SDX-103	500	SDX-103	400	27.1 ± 1.6 (8)	131%*	0	-5.4%; Day 4	2
4	10	SDX-103	300			26.6 ± 1.0 (10)	129%*	0	-2.9%; Day 2	0

Table 3. Treatment Response for P388. Both treatments with SDX-103 at 300 mg/kg and 500-400 mg/kg caused a statistically significant increased in mean survival (p=0.0059 and 0.0016 respectively, t-test; p<0.01 for both ANOVA). Paclitaxel treatment also provided a statistically significant increased in mean survival. * Statistically significant from control, p<0.01 ANOVA.
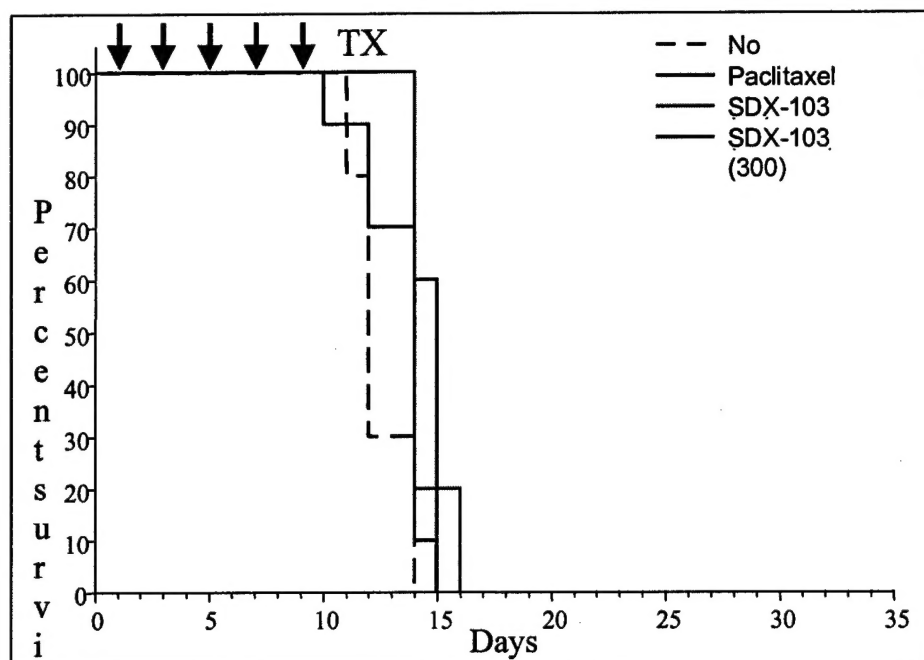
Figure 1. Kaplan-Meier Survival Plot for the P388 Study. The efficacies of paclitaxel and SDX-103 regimens are illustrated by the Kaplan-Meier survival curves. Two deaths occurred in the 500 mg/kg group ay day 2 and day 7, the dose was then reduced at 400 mg/kg for the last two injections at day 7 and 9.

The results with the multidrug resistant P388/ADR model were disappointing (Table 4 and Figure 2). Although the T/C ratio was significantly higher in the treated animals, the overall value (119%) was below the "activity" threshold defined by the NCI (120%) and the drug should have been considered inactive.

| Group | n | Primary | | Secondary | | Mean Day of Survival | %T/C | # 30 Day Survival | Max BW. Loss | # of Toxic |
		Agent	mg/kg	Agent	mg/kg	Mean ± SEM (n)			%; (Day)	Deaths
1	10	No Treatment	n/a			12.4 ± 0.4 (10)	100%	0	---	0
2	10	Paclitaxel	20			13.3 ± 0.5 (10)	107%	0	-1.0%; Day 2	0
3	10	SDX-103	500	SDX-103	400	14.8 ± 0.2 (10)	119%*	0	-5.0%; Day 2	0
4	10	SDX-103	300			14.2 ± 0.1 (10)	115%*	0	-2.5%; Day 2	0

Table 4. Treatment Response for indanocine phosphate in the P388ADR model. Paclitaxel treatment did not show a significant increase of survival ($p > 0.05$, ANOVA). Both treatments with SDX-103 at 300 mg/kg and 500-400 mg/kg caused a small, but significant increase of survival ($p < 0.0001$ and $p = 0.0002$ respectively, t-test; $p < 0.01$ for both ANOVA). * Statistically significant from control, $p < 0.01$ ANOVA

Figure 2. Kaplan-Meier Survival Plot for the P388 Study. The efficacies of paclitaxel and SDX-103 regimens are illustrated by the Kaplan-Meier survival curves. Although the survival curves for the four groups have similar step slopes, the first deaths from tumor progression occurred on Day 12 in the untreated and paclitaxel-treated groups, whereas both SDX-103 treatments delayed the first deaths until day 14.
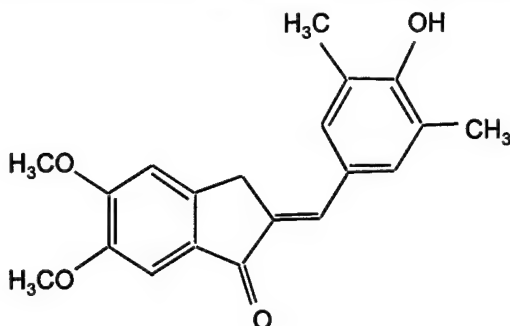
Overall, the results with these murine models were somewhat disappointing, since the T/C ratio obtained was modest, and the doses used were high when compared to the in vitro results and when compared to other established anti-cancer agents.

Further analysis of the pharmacokinetic and pharmacodynamic properties of Indanocine-phosphate should allow us to understand the reasons of the poor in vivo activity. One possibility is that the compound is rapidly de-phosphorylated and drops out of solution, becoming inactive. Another possibility is that a rapid metabolic inactivation occurs via glucoronidation of the hydroxy residue. Both hypothesis will be tested as soon as a bioanalytical assay for the plasma detection of Indanocine will be developed.

B) Results with the indanocine analog NSC692745

Concomitantly with the assays using Indanocine-phosphate, we initiated a collaboration with the National Cancer Institute for the testing of an Indanocine analog (lacking an amine group) named indanorine (NSC692745 by NCI nomenclature, figure 1). Indanorine was selected because of preliminary in vivo results at the NCI using hollow-fiber models demonstrated its superiority compared to Indanocine (data not shown).

Several different doses, schedules and formulations were tested at the NCI using human cancer cell

lines injected in athymic nude mice (Xenograft models Table 1).

Cell line	Tumor	Formulation	Schedule, implant site	Doses (mg/kg/dose)	Number animals	Results and comments
MDA-MB-435	Breast cancer	30%HPCD, pH 8.0	Q4Dx3, Day 27, IP	0 90 134 200	18 9 9 9	MTD achieved (~200 mg/kg/dose), no significant anti-tumor activity
NCI-H522	Non-small cell lung cancer	30%HPCD, pH 8.0 (cyclodextranes)	QDx5, Day 11, IP	0 54 80 120	12 5 5 5	MTD achieved (~60 mg/kg/dose), anti-cancer activity observed at 54 mg/kg/dose
OVCAR-3	Ovarian carcinoma	10%DMSO in Saline/Tween 80	Q4Dx3, Day 61, SC	0 79 117 175	20 10 10 10	Poor tumor growth in control animals
LOX IMVI	Melanoma	10% DMSO/90%PEG 300	QDx5, Day 1, IP	0 54 80 120	6 6 6 6	No activity. The vehicle used was toxic

Table 5: NCI Xenograft Models. Summary of the in vivo testing performed at the NCI with the Indanocine analog NSC692745. The assays have been performed at the National Cancer Institute, Division of Cancer Treatment, Development Therapeutics Program, Biological Testing Branch between June 1999 and July 2000.

As one can easily see from the NCI experience, the identification of the optimal route or delivery, dosing schedule, formulation and total dose is a very labor intensive task.

Overall, we have underestimate the issues and problems related to the development of a novel anti-cancer agent. Our current intention is to pursue a "rational" development of indanocine (or a closely related analog), optimizing the experimental conditions to reduce the time and costs of the

development of the molecule. We strongly believe that the new results presented in this updated report demonstrate that we did our best to accomplish the specific aims listed in Task 2. We also hope that the reasons for the deviations from the original proposed research plan would be more easily understood.

At the end of 2001, a start-up company (Salmedix, Inc) licensed the Indanocine patents and is now currently developing indanocine (and related analogs) with the intent of filing an investigational new drug (IND) application to the FDA by the end of 2003. This may allow the initiation of clinical trials with Indanocine during 2004. Salmedix plans to identify the ideal Indanocine candidate (either a formulation or an analog) and perform the ADME and toxicology studies required for the IND application. Salmedix will also optimize the synthetic process, produce cGMP quality material, and complete additional in vitro pharmacology studies (including PK and tumor efficacy) required for the initiation of clinical trials.

Reportable Outcome

Articles:

Hua XH, Genini D, Gussio R, Tawatao R, Shih H, Kipps TJ, Carson DA, Leoni LM. Biochemical genetic analysis of indanocine resistance in human leukemia.Cancer Res. 2001 Oct 1;61(19):7248-54.

Leoni LM, Hamel E, Genini D, Shih H, Carrera CJ, Cottam HB, Carson DA. Indanocine, a microtubule-binding indanone and a selective inducer of apoptosis in multidrug-resistant cancer cells. J Natl Cancer Inst. 2000 Feb 2;92(3):217-24.

Giannakakou P, Sackett D, Fojo T. Tubulin/microtubules: still a promising target for new chemotherapeutic agents. J Natl Cancer Inst. 2000 Feb 2;92(3):182-3.

Abstracts:

Leoni LM, Hua XH, Tawatao R, Wei J, Kipps TJ, Carson DA. In vitro and in vivo anticancer activity of SDX-103, a water-soluble prodrug of indanocine that is selectively toxic to leukemic cells. Proceedings American Association for Cancer Research Annual Meeting. 2002 March; 43:266.

Hua XH , Leoni LM, Shih H, Carrera CJ, Carson DA. Induction of apoptosis by microtubule disrupting drugs in chronic lymphocytic leukemia. Blood. 2000 November 16; 96:169a.

Patents:

Carson DA, Shih HC, Cottam HB, Leoni LM. Inadone and tetralone compounds for inhibiting cell proliferation. Patent Number US 6162810. Granted December 19, 2000

Previous Reports

For the benefit of the reviewers I included the previous reports that were submitted to the attention of the DOD. Taken together, including the published papers and the patent, this document includes all the results obtained with the support of the DAMD17-99-1-9100 grant.

Report Year 2000

Task 1

In the first aim of our application, we proposed to characterize further the mechanism of action and the molecular targets of indanocine and related compounds in multidrug resistant breast cancer cell lines. Specifically, we planned to:

5. Develop indanocine resistant breast cancer cell line mutant and assess its pattern of cross resistance to other agents (months 1-24)
6. Characterize biochemically the resistant phenotype, emphasizing cytoskeletal and apoptotic regulatory proteins (months 7-18)
7. Investigate differentially expressed genes in the resistant mutants by human cDNA expression chips (months 12-24)
8. Test the role of the differentially expressed genes by transfection in the wild type cell lines (months 24-32)

Point #1
Establishment of an indanocine resistant cell line

We obtained an indanocine-resistant clone (CEM-178) derived from the human T lymphoblastoid cell line CEM. The CEM-178 cells can proliferate in presence of up to 300nM of indanocine. The resistant phenotype persisted when the mutant cells were cultured in drug-free medium for at least 9 months. The doubling time of the CEM-178 was 24 hours as compared to 18 hours of the parental cells. Major morphological alterations, for example, size or shape, were not observed. Quantitative analysis of DNA content by flow cytometry did not reveal any change. Karyotyping also showed no difference between CEM and CEM-178 (data not shown).

The drug sensitivity of parental WT CEM and indanocine-resistant clone CEM-178 is shown in Table 1.

Table 1: Profile of chemosensitivity of the indanocine-resistant cells CEM-178

Drugs	CEM IC_{50}	CEM-178 IC50	Fold
indanocine	1.79 nM	206 nM	115
paclitaxel	0.5 nM	0.5 nM	1
colchicine	2.0 nM	62.5 nM	31
vinblastine	0.1 nM	4.0 nM	40
fludarabine	5.8 µM	13.3 µM	2.3
doxorubicin	0.2 µM	0.4 nM	1.9
cytochalasin B	7.1 µM	4.6 µM	0.6

The CEM-178 cells displayed cross-resistance to other tubulin-depolymerizing drugs, such as vinblastine (40 fold) and colchicine (31 fold) but not to the tubulin-polymerizing agent paclitaxel. No cross-resistance to other anti-tumor drugs like fludarabine, doxorubicin and cytochalasin B was observed.

Point #2
Biochemical characterization of the resistant cells

We performed an extensive biochemical characterization of the CEM-178 cells, by measuring the expression of the several proteins involved in the apoptotic regulation.
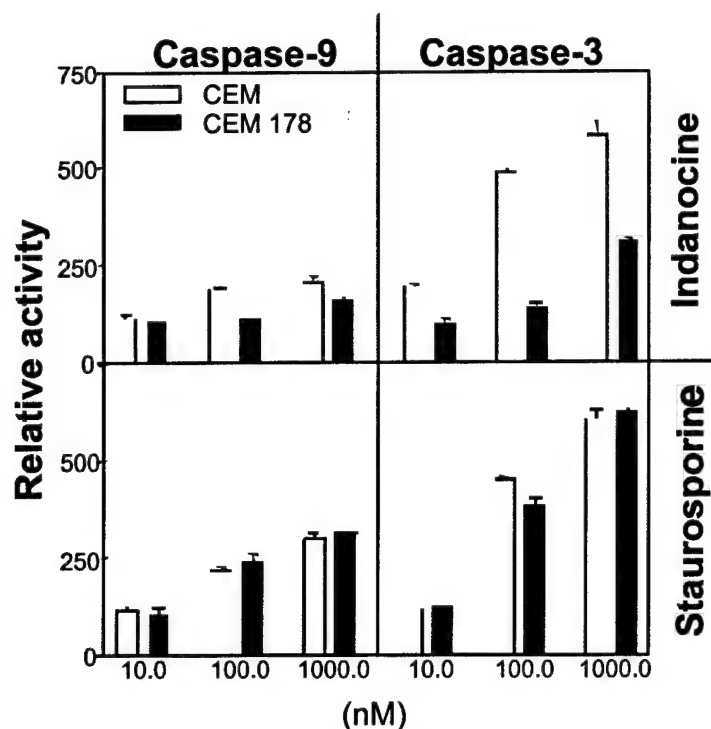
Figure 1: Activity of caspase-3 and –9 in indanocine and staurosporine-treated wild type CEM and CEM-178 cells.

Figure 1 show the pattern of caspase activation of the CEM-178 as compared to CEM treated with indanocine and staurosporine. The results indicate that the CEM-178 cells cells displayed a lower level of caspase-3 and –9 activity when treated with indanocine, but retain a functional apoptotic machinery as indicated by activation of caspases when treated with staurosporine. Indanocine failed to induce apoptosis in the CEM-178 cells, as indicated by the lack of cytosolic cytochrome c release (Fig 2)

Figure 2: Indnanocine-induced cytochrome c cytosolic release in indanocine-treated CEM and CEM-178 cells

Additional biochemical assays, that can not be detailed for reason of space, suggested that the CEM-178 expressed tubulin with altered polymerization/depolymerization properties. In order to investigate the tubulin alterations on the CEM-178 cell we sequenced the 2 predominant isotypes of tubulin, M40 and b2, in both parental WT CEM and CEM-178 cells.

One point mutation was identified in the cDNA of M40 isotype, with a G to T substitution at nucleotide 1050, which converts amino acid 350 from lysine (AAG) to asparagine (AAU). This substitution was present at a single peak suggesting that there was no expression of the wild type sequence. Molecular modeling experiments performed in collaboration with Rick Gussio at the National Cancer Institute suggests that the observed tubulin mutation (K→D) may hinder the binding of indanocine (and cochicine) to tubulin, without affecting the capacity of tubulin to polymerize or depolymerize. Detailed studies on the modeling of the indanocine/tubulin interactions are currently underway, but it is already clear the CEM-178 cells will be very helpful in defining the molecular details of the mechanism of action of several other clinically used anti-cancer drugs.
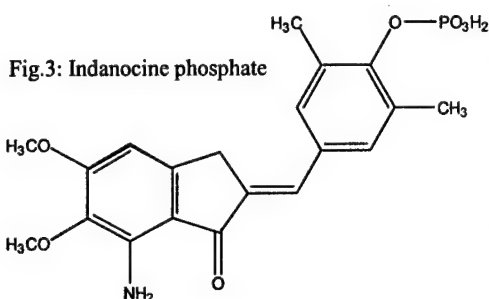
Point 3 and 4

We are currently preparing an experiment to look at the differences in gene expression between the CEM and CEM-178 cells using gene arrays. We also are cloning the mutated tubulin isoform found in the CEM-

178 cells into a mammalian expression vector. We plan to produce stable cells expressing the altered tubulin to verify its role in indanocine resistance.

Task 2

In the second aim of the application we proposed to assess the in vivo antitumor activity of indanocine in nude mice bearing multidrug resistant human breast cancer cell lines. Specifically, we planned to:

4. Determine the acute and chronic toxicity of indanocine and related compounds in nude mice.
5. Establish human tumor models in nude mice using wild type, multidrug resistant, and indanocine resistant breast cancer cell lines.
6. Test indanocine and related compounds in the established in vivo tumor models.

Fig.3: Indanocine phosphate

The main problem we encountered when testing indanocine in animals was the poor water solubility of the compound. Indanocine can only be prepared in DMSO solutions at a concentration of up to 20 mM concentrations. The highest concentration achievable in acquesous solutions is 100 µM. In order to overcome this problem we generated an indanocine analog that can be solubilized in water and other acqueous solutions (Fig. 3).

We used indanocine-phosphate in all the experimenst performed in animals.

Recent studies suggest that tubulin-binding drugs may display in vivo anti-cancer activity by reducing the blood supply to the cancer cells (anti-vascular activity), and by selectively killing cells on the blood vessels that feed the tumot cells (anti-angiogenic activity).

Calculated IC50

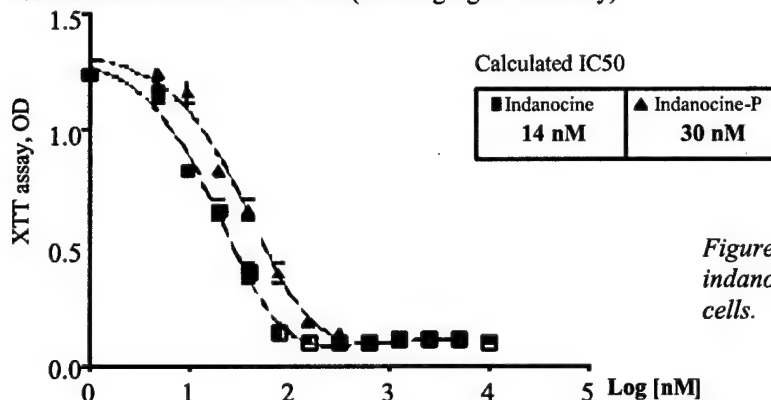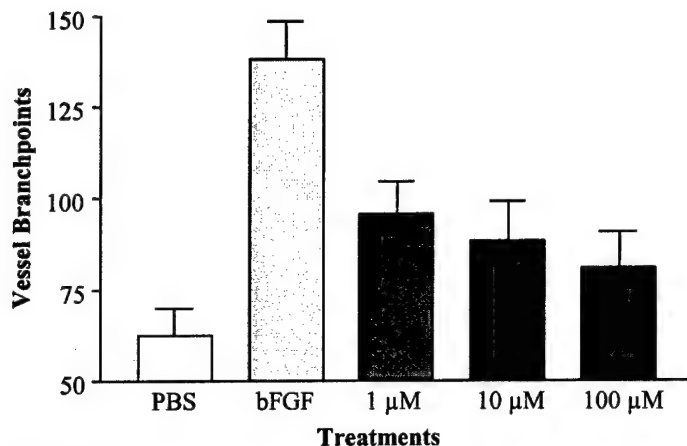| ■ Indanocine	▲ Indanocine-P
14 nM	30 nM

Figure 4: Cytotoxic activity of indanocine-phosphate in HUVEC cells.

We first tested the cytotoxic activity of indanocine and indanocine-phosphate against human umbilical vein endothelial cells (HUVEC), using the MTT proliferation assay (Figure 4). We obtained an IC50 of 14 nM for indanocine and 30 nM for indanocine-phosphate.
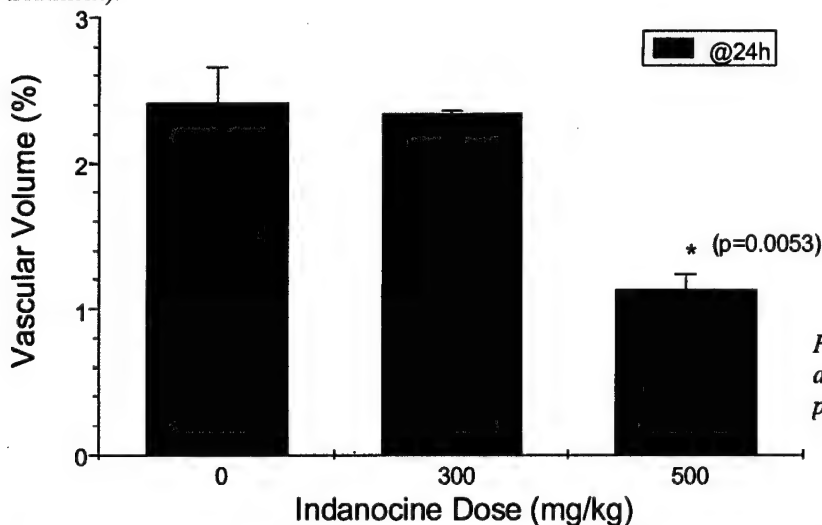
In collaboration with Judy Varner at UCSD Dept. Medicine, we then tested the in vivo anti-angiogenic activity of indanocine in the the chick chorioallantoic membrane (CAM) model (Figure 5).

Figure 5: Anti-angiogenic activity of indanocine-phosphate tested in the CAM assay

Indanocine-phosphate was able to reduce the number of vessel branchpoints induced by fibroblast growth factor beta (bFGF), without displaying any visible toxicity to the chicken embryo. The results obtained clearly indicate that indanocine possess anti-angiogenic activity.
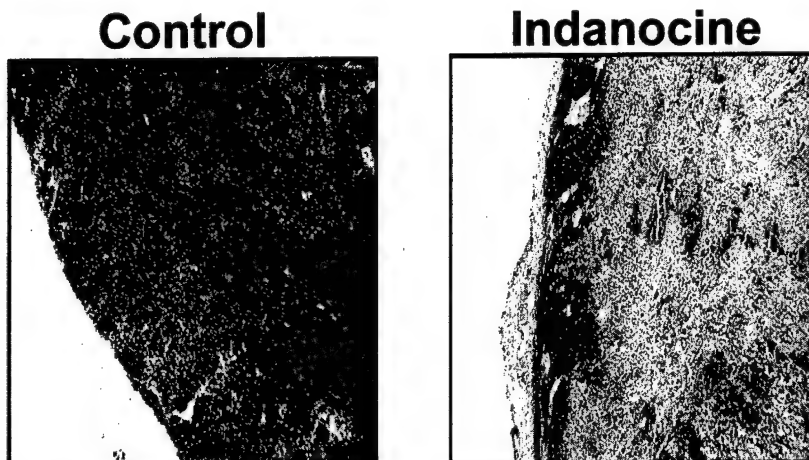
In order to further investigate the in vivo activity of indanocine, we then initiated a collaboration with Dr. Charles Parkins at the Tumour Microcirculation Group at the Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Northwood, England. We used the very well established murine breast adenocarcinoma CaNT tumour-bearing CBA female mice model. Indanocine-phosphate is very well tolerated by the animals even at very high concentrations (>500 mg/kg). The data we obtained show that there is no significant cytotoxicity by indanocine-phosphate against cultured CaNT tumour cells and very slight cytotoxicity against tumours treated in vivo (assayed using excision assay at 24h after treatment).

Figure 6: Anti-vascular activity of indanocine-phosphate

There was evidence for a significant anti-vascular action of indanocine using the vascular volume assay at 500mg/kg after 24 hours (**Fig X+3**). In order to confirm the anti-vascular activity of indanocine we performed histological analysis (**Fig X+4**). The pictures show that only a viable rim of tumor cells survive, all cells within the central part of the tumour have been destroyed by indanocine-phosphate. The results obtained are very smilar to those observed with other anti-vascular agents, such as combretastatin A-4.

Control   Indanocine

Figure 7: Indanocine induces selective tumor cell death in vivo

Taken together these preliminary results indicate that further detailed investigations on the anti-angiogenic and anti-vascular activity of indanocine are warranted. The major problems that are expected are the bioavailability of the drug and its delivery route. Preliminary assays suggest that most of the drug seem to be protein-bound, and that the half-life of the drug in vivo is very short. We plan to address these issues by a medicinal chemistry approach and by combination of the drug with carrier molecules. Synthesis of novel analogs with a better pharmacological profile and co-delivery of indanocine with lipids will be performed within one year.

Reportable Outcome

1. Manuscript JNCI

Leoni, Lorenzo M.; Hamel, Ernest; Genini, Davide; Shih, Hsiencheng; Carrera, Carlos J.; Cottam, Howard B.; Carson, Dennis A. Indanocine, a microtubule-binding indanone and a selective inducer of apoptosis in multidrug-resistant cancer cells. In: Journal of the National Cancer Institute (Bethesda) Feb. 2, 2000. 92 (3): 217-224.

2. Patent on Indanocine

09/148,576 Carson, Leoni, Cottam and Shih: Novel Anticancer Agents. Claims indanocine and related agents. US Patent Application, European Patent Application # 98958023.8-2112

Report Year 2001

Introduction

Mechanism of Action of Substituted Indanones in Multidrug Resistant Breast Cancer

Our laboratory has recently synthesized a series of novel substituted indanones that are selectively toxic to multidrug resistant cancer cells, including breast cancer cell lines.
In this application we proposed to characterize the mechanism of action of indanocine and to assess the in vivo anti-tumor activity of indanocine.
During the second year we:
- published the second report on the biological activity of indanocine (Cancer Res 2001 Oct 1;61(19):7248-54)
- analyzed the indanocine-resistant stable cell line
- identified the potential indanocine-binding site on tubulin
- continued the animal testing of indanocine
- studies the pro-apoptotic mechanism of action in non-dividing tumor cells

The results shown in this annual summary demonstrate that indanocine is a very promising new anti-cancer agent, with selective activity in slowly-dividing or quiescent tumor cells. The positive early animal models suggest that indanocine could be soon ready for clinical trials

Body

Task 1
In the first aim of our application, we proposed to characterize further the mechanism of action and the molecular targets of indanocine and related compounds in multidrug resistant breast cancer cell lines. Specifically, we planned to:

9. Develop indanocine resistant breast cancer cell line mutant and assess its pattern of cross resistance to other agents (months 1-24)
10. Characterize biochemically the resistant phenotype, emphasizing cytoskeletal and apoptotic regulatory proteins (months 7-18)
11. Investigate differentially expressed genes in the resistant mutants by human cDNA expression chips (months 12-24)
12. Test the role of the differentially expressed genes by transfection in the wild type cell lines (months 24-32)

Almost all of the specific aims that we planned to investigate in this grant have been addressed by our recently published in Cancer Research (Cancer Res 2001; 61:7248-54). Since the published manuscript is included as an appendix to this annual report, I will refer to it for the experimental details and the major findings.
The most important result in the manuscript is the identification of a tubulin mutation that confers resistance to indanocine in the selected cells. The observed mutation has allowed us to identify a novel tubulin-binding site of indanocine. We plan to use the information generated by these studies to optimize the synthesis of novel indanocine analogs using computer-aided modeling in collaboration with the medicinal chemistry group of Dr. Howard Cottam at UCSD.

The Cancer Research manuscript also reports the surprising finding that indanocine is selectively toxic to non-dividing primary leukemic cells isolated from chronic lymphocytic leukemia (CLL) patients. Since we previously demonstrated that indanocine was able to induce apoptosis in quiescent multidrug resistant breast cancer cell lines (see J Nat Canc Inst, 2000. 92: 217-224), we decided to start studying the killing mechanism in primary tumor cells.

Any anti-cancer agent that is able to selectively induce cell death to non-dividing or slowly dividing tumor cells would represent a great weapon in the cancer chemotherapy arsenal. Indanocine may be used to kill the "quiescent" subset of cancer cells that may not be affected by the conventional chemotherapy.

In the following section we demonstrate the pro-apoptoptic activity of indanocine in primary tumor cells, and we propose a mechanism of action for the killing of non-dividing cells.

When quiescent primary tumor cells are incubated with indanocine, a rapid and potent apoptotic program was activated. Loss of mitochondria transmembrane potential was observed as indicated by the reduction of the DiOC6 staining (Figure 1).
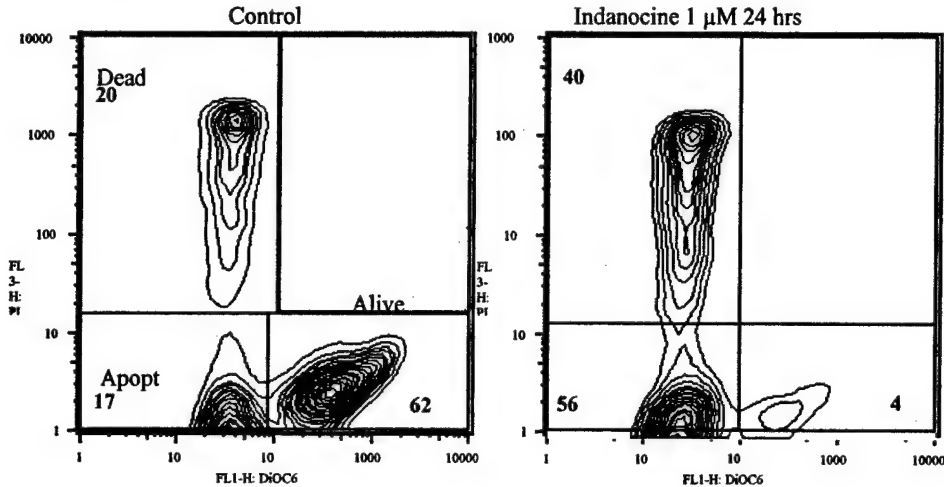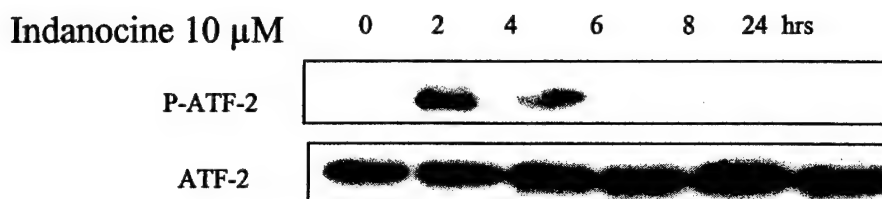

Figure 1. Selective induction of apoptotsis by indanocine in primary tumor cells. Cells were then double stained with DiOC6 and PI, and subject to flowcytometry analysis.

Caspase-3 was rapidly activated after only 2 hour of incubation time point (Figure 2), concomitantly to cytochrome c release from its mitochondrial compartment into the cytosol (dato not shown).
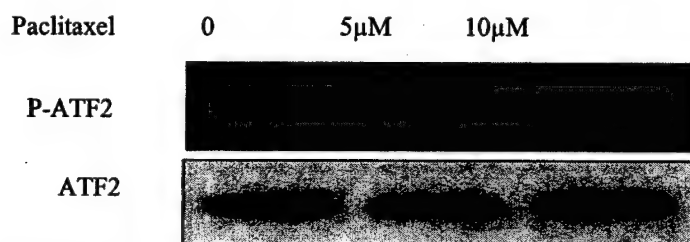

Figure 2. Time course of caspase-3 cleavage. CLL cells were incubated for indicated periods of time with 10μM of indanocine. The cells were then lysed. Cleaved caspase-3 was visualized on Western Blotting using an antibody specific for active caspase-3.

In order to identify the early events activated by indanocine responsible for the activation of the apoptotic program, we investigated the role of the p38 kinase signaling cascade. The p38 kinase has been shown to play an important role in inducing apoptotic cell death. p38 kinase is activated by phosphorylation by the MEK3/6 kinases. The active p38 can phosphorylated and activate transcription factors, such as ATF2. p38 kinase may also activate apoptosis in an transcriptional independent mechanism affecting Bcl-2 family members such as Bax, or facilitating cytochrome c release from mitochondria. In order to test whether p38 kinase was activated by indanocine, we used ATF2 phosphorylation as a surrogate marker of p38 kinase activity. We showed that ATF-2 was phosphorylated at 2 hours following indanocine incubation (Figure 3).

Indanocine 10 μM   0   2   4   6   8   24 hrs

P-ATF-2

ATF-2

Figure 3. Time course of ATF-2 phosphorylation in tumor cells.
Phosphorylation of ATF-2 was visualized by Western Blotting using phosphospecific antibodies (Cell
Siganling Inc). Total ATF-2 protein was also shown as control.

The ATF-2 phosphorylation was not observed when normal lymphocytes were incubated with indanocine.
Since indanocine do not induce apoptosis in normal lymphocytes, a correlation between killing and ATF-2
phosphorylation was established. This correlation was further corroborated by the finding that paclitaxel, a
potent microtubule binding agent, was unable to kill the indanocine-sensitive primary malignant leukemic
cells and was also unable to induce ATF-2 phosphorylation (Figure 4).

Paclitaxel   0   5μM   10μM

P-ATF2

ATF2

**Figure 4. Paclitaxel do not induce ATF-2 phosphorylation in primary
leukemic cells.** Phosphorylation of ATF-2 was visualized by Western Blotting using
phosphospecific antibodies (Cell Siganling Inc). Total ATF-2 protein was also shown as control.

p38 kinase can be phosphorylated and activated by MEK3/6, which is a MAPK kinase (MAPKK). In turn,
MEK3/6 can be phosphorylated by mixed lineage kinases (MLK), including the apoptotic signal-regulating
kinase 1 (ASK1). Over expression of ASK1 causes apoptotic cell death, and kinase-inactive form of ASK1
blocks the apoptosis induced by tumor necrosis factor-alpha. We discovered that indanocine can activate
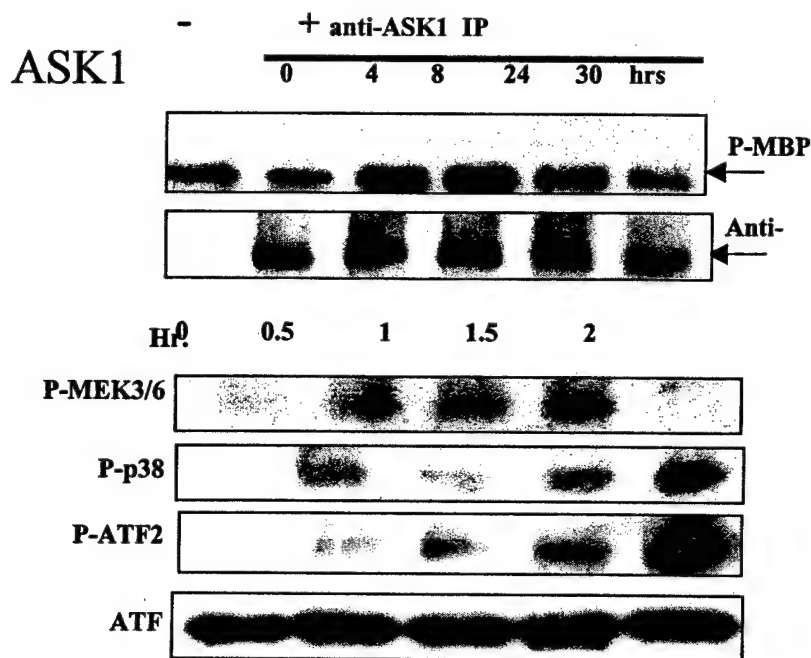the ASK1-MEK3/6-p38 kinase cascade (Figure 5.)

Figure 5. Indanocine activates the ASK1-MEK3/6-p38 kinase-ATF2 signaling cascade. The measure ASK1 activity, cell lysates were subject to immunoprecipitation followed by in vitro kinase assay using MBP as substrates. MBP was strongly phosphorylated at 4 hour. Anti-ASK1 Western blot was also performed to show equal amounts of ASK1 was precipitated. For the detection of MEK3/6, p38 kinase and ATF2, phospho-specific antibodies were used (Cell Signaling Inc).

In living cells, ASK1 is inhibited by thioredoxin. The oxidation of thioredoxin by reactive oxygen species can causes its dissociation from ASK1 and may result in the induction of ASK1 kinase activity. To test this hypothesis we analyzed the levels of reactive oxygen species (ROS) in primary tumor cells after incubation of indanocine. The results we obtained indicate that indanocine can rapidly increase the ROS in the tumor cells (Figure 6).
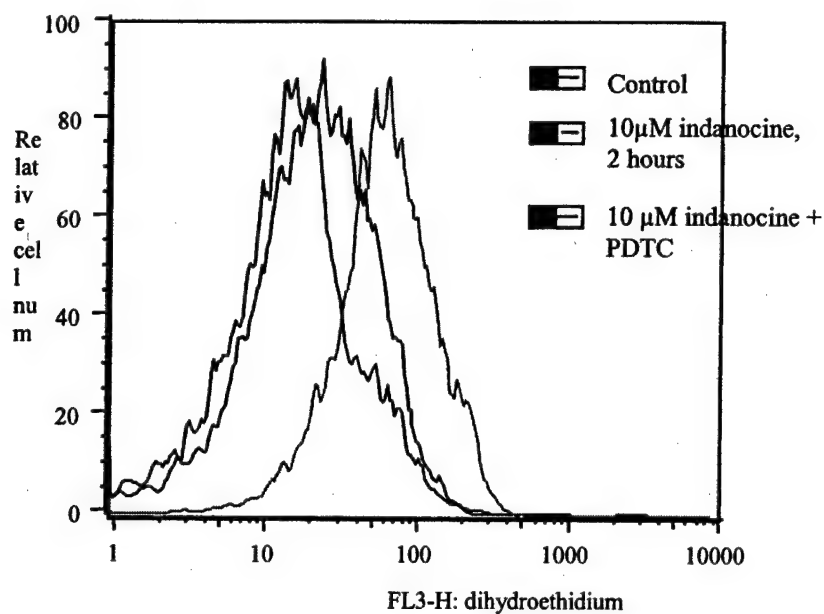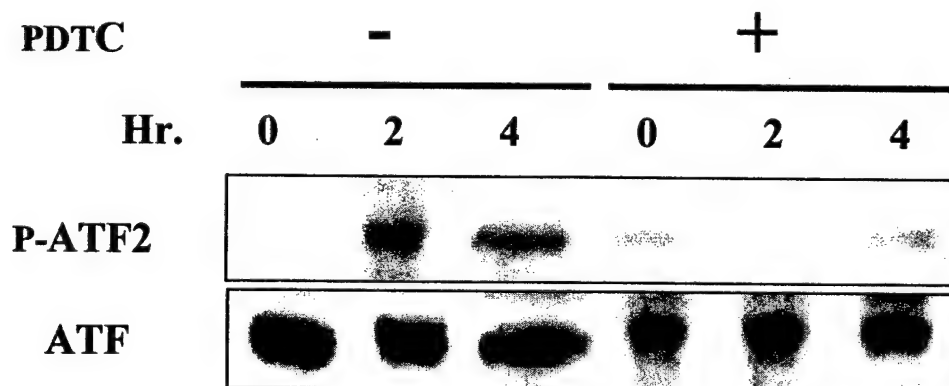
FL3-H: dihydroethidium

Figure 6. Indanocine increased intracellular O2- production in primary tumor cells.
Tumor cells were incubated with medium alone, 10μM of indanocine, or 10μM indanocine with PDTC for 2 hours. The generation of O2- was quantified by staining the cells with dihydroethidium and analysed by flow cytometry.

In addition, the prevention of ROS formation using the free radical scavenger, Pyrrolidine dithiocarbamate (PDTC), was able to prevent the indanocine-induced ATF2 phosphorylation (Figure 7).

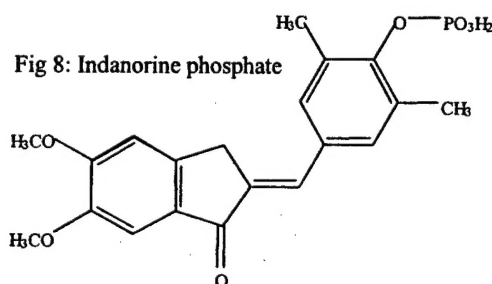Figure 7. Inhibition of O2- production prevents ATF2 phosphorylation.

Task 2

In the second aim of the application we proposed to assess the in vivo antitumor activity of indanocine in nude mice bearing multidrug resistant human breast cancer cell lines. Specifically, we planned to:

7. Determine the acute and chronic toxicity of indanocine and related compounds in nude mice.
8. Establish human tumor models in nude mice using wild type, multidrug resistant, and indanocine resistant breast cancer cell lines.
9. Test indanocine and related compounds in the established in vivo tumor models.

The main problem we encountered when testing indanocine in animals was the poor water solubility of the compound. Indanocine can only be prepared in DMSO solutions at a concentration of up to 20 mM concentrations. The highest concentration achievable in acqueous solutions is 100 μM. In order to overcome this problem we generated an indanocine analog that can be solubilized in water and other acqueous solutions (Fig. 8).

Indanorine phosphate can be solubilized in water solutions at concentrations up to 100 mg/ml. The *in*

Fig 8: Indanorine phosphate

vitro activity of indanorine phosphate to cancer cell lines was similar, although reduced, to the parenteral compound, indanocine. The bulky and negatively charged phosphate group of indanorine phosphate do not allow the duect penetration through the cell membrane barrier. The difference between the two compounds may be due to the dephosphorylation kinetics.

Cell line	Indanorine phosphate	Indanocine
	IC_{50}, nM	IC_{50}, nM
Jurkat	15	5
MOLT-4	17	7
MCF7	45	20
MCF7/ADR	16	4
MDA-MB-321	35	10
HCT-116	100	55
VM46	100	50
AZ2780	45	30
AZ2780/CD10	30	15

Table 1. Growth-inhibitory activity of indanocine and indanorine phosphate in tumor cell lines. The cells were treated with various concentrations of the indicated drugs for 72 hours. Cell proliferation was assessed with the MTT assay. The results represent the 50% growth-inhibitory concentrations (IC_{50})

Indanorine phosphate also retained its activity against primary quiescent malignant lymphocytes (data not shown).

We used indanorine phosphate for our in vivo assays.

<u>Maximum Tolerated Dose in Athymic Nude Mice</u>

The toxicity of indanorine phosphate was determined in two stages.
(1) The dosage range for the MTD study was determined in a one-mouse dose-escalating study. The drug was administered i.v. in a single dose to a single animal. The dose was then doubled successively for each of the following animals. (2) The MTD study employed two routes of administration, two dosing schedules, and three dosing levels and required twelve groups of mice.
The MTD study indicated that Indanorine phosphate was well tolerated by the athymic nude mice. No MTD was achieved (following NCI guidelines) at concentrations of 100, 200, and 300 mg/kg given i.v. or i.p. at five daily doses (QD x 5) or at five doses given on alternate days (QOD x 5).

<u>In Vivo Evaluation of Indanorine Phosphate against the P388 Murine Leukemia Lines</u>

To evaluate the in vivo efficacy of indanorine phosphate, we tested indanorine phosphate in the P388 murine leukemia model. Female B6D2F1 mice were inoculated on Day 0 with 1 x 106 P388 leukemia cells harvested from tissue culture. As a positive control we used i.p. paclitaxel at 25 mg/kg on a qod x 5 schedule. Indanorine phosphate was dosed at 300 and 500 mg/kg 5 doses QOD. Toxicity was observed at 500 mg/kg and the dose was lowered at 400 mg/kg for the last 3 doses.

Group	n	Primary				Secondary			
		Agent	mg/kg	Route	Schedule	Agent	mg/kg	Route	Schedule
1	10	No Treatment	n/a						
2	10	Paclitaxel	20	IP	QOD x 5				
3	10	SDX-103	500	IP	D 1, 3, 5	SDX-103	400	IP	D 7, 9
4	10	SDX-103	300	IP	QOD x 5				

Table 2. Protocol design of P388 animal model.

Table 3. Summary of the results of P388 animal model.

Group	n	Primary		Secondary		Mean Day of Survival	%T/C	# 30 Day Survival	Max BW. Loss	# of Toxic
		Agent	mg/kg	Agent	mg/kg	Mean ± SEM (n)			%; (Day)	Deaths
1	10	No Treatment	n/a			20.7 ± 1.3 (10)	100%	0	---	0
2	10	Paclitaxel	20			27.1 ± 0.8 (8)	131%	0	---	0
3	10	SDX-103	500	SDX-103	400	24.2 ± 1.3 (5)	117%	0	-5.4%; Day 4	2
4	10	SDX-103	300			26 ± 0.8 (9)	126%	0	-2.9%; Day 2	0

Since the NCI cut-off for anti-leukemia activity of an agent in the P388 model is a 120% T/C value or a 20% increase in life span (%ILS), Indanorine phosphate has to be considered active at a dose of 300 mg/kg (T/C 126%). Unfortunately, The T/C ratio is not as high as we may have expected based on the in vitro results.

More assays are currently underway to assess the activity of Indanorine phosphate. In particular we are testing the possibility that indanorine phosphate may have a very short half-life, and therefore, may have to be delivered more frequently. In the current assays we are dosing the drug daily (QD) and twice daily (BID) at 300 mg/kg.

If the results of the current animal assays will be positive we plan to initiate pharmacokinetic and pharmacodynamic studies. Tumor Xenografts in Athymic Nude Mice using human breast cancer cells will then follow, as indicated in the original proposal.

We do not plan to futher pursue assays to determine the in vivo anti-angiogenic or anti-vascular activity of indanocine, before obtaining positive results in the "conventional" tumor models.

Negative results on the new P388 assays (i.e. no increaese in the T/C ratio), will prompt us to test new indanocine analogs that have been prepared for us by the group of Dr. Cottam at UCSD.

Reportable Outcome

Manuscripts
Cancer Res 2001 Oct 1;61(19):7248-54. Biochemical genetic analysis of indanocine resistance in human leukemia.Hua XH, Genini D, Gussio R, Tawatao R, Shih H, Kipps TJ, Carson DA, Leoni LM.

Patent
09/148,576 Carson, Leoni, Cottam and Shih: Novel Anticancer Agents.   Claims indanocine and related agents. US Patent Application, European Patent Application # 98958023.8-2112